Understanding the Effects of Polydispersity on Protein Conjugation

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Jacob Benjamin Gerstein

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Abstract

The level of polydispersity or aggregation in a protein population is an important factor to consider when performing conjugation or labeling reactions. In such reactions, the more aggregated portions of a polydispersed protein population have a tendency to be less labeled—or have a lower degree of labeling (DOL)—than the more highly labeled, less aggregated portions of the population. The mechanism by which this differential labeling occurs is thought to work as follows: When conjugating a protein to a detection mechanism or other conjugate of interest, the reaction, as it occurs in solution, can be visualized as taking place in a vast, empty space. In such a scenario, each particle in the reaction is very dilute relative to the total volume of the reaction. Therefore, each protein particle in the reaction has a similar, average number of chemical interactions with the conjugate molecules in the reaction, regardless of the protein particle's size or number of subunits. The number of molecular interactions a particle will have during the conjugation reaction is very unlikely to be affected by the number of subunits it contains; conversely, however, the number of subunits will have a considerable effect on the DOL calculated for a given set of the dispersed population.

If we assume the monomeric portion of a dispersed protein population receives an average of one label during conjugation, then we would also assume that the more aggregated portion of the population will receive the same average number of labels and have the same number of labels *per particle*. However, because the two sets of particles (monomeric and aggregated) do not contain the same number of *subunits*, the degree of

labeling will differ quite significantly. This is because the amount of protein present is inversely related the degree of labeling; as the protein concentration increases, the degree of labeling decreases.

The study described in this thesis seeks to demonstrate that the different polydispersed populations present in a conjugate solution will have significant differences in their DOL with the smaller, less aggregated particles in the conjugate having a DOL than larger, more aggregated particles. To do this, two special batches of fluorescent protein conjugates were prepared, one labeled with Alexa Fluor 594 and the other with Alexa Fluor 488. Both of these conjugates were fractionated by size and the fractions of interest were promptly assayed to determine their DOL and to compare the level of labeling in each of the polydispersed populations in the conjugate sample. The data collected for both prepared conjugates clearly showed that there were considerable differences in DOL and a clear trend indicating that the DOL of each species in a polydispersed conjugate population decreased as the level of aggregation, or number of protein subunits per protein, increased.

Acknowledgments

I would like to acknowledge the contributions of my colleagues Aaron Handler and Rick Chu, whose guidance on this project was truly invaluable.

Dedication

This work is dedicated to my wife, who supported me unequivocally throughout this journey.

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Chapter I

Introduction

Ligand-binding Assays in Biotech Research:

When a patient is dosed with a biotherapeutic drug, it is critical for researchers and physicians to understand the downstream effects of that treatment on the body in as much detail as is possible. Studies of drug metabolism are known as pharmacokinetic (pK) studies and are performed to examine the absorption, distribution, break-down, and excretion of the drug under investigation. Understanding the relationship between the size of a dose, its concentration throughout the body and at the intended site of action, and the eventual clearance of the drug from the body are all part of the pK assessment. The data obtained from a pK study is often useful for the design and format of later clinical trials, as understanding the drug's absorption and distribution in the body after dosing is also very helpful when trying to interpret efficacy and safety data generated. The results of pK studies can also be of use in determining which patients may be the best candidates to benefit from a particular drug treatment.

Understanding pharmacokinetic drug actions in relation to the characteristics of a patient's disease state, or the tissue where the disease is presenting, may have some relevance to the eventual success or failure of the treatment. Therefore, it is always important to evaluate the efficacy of the investigational drug, or the nature of any adverse reactions observed during a clinical trial, in relation to the physiological characteristics of each individual study participant, whether they are a patient or a healthy volunteer (Japanese Ministry of Health, 2001).

A critical parameter of pK studies is the accurate measurement of drug concentrations in the patient's serum after dosing. To measure the concentration of a biotherapeutic drug in a complex protein matrix such as serum, the assay used must have the ability to detect the presence of the drug without background interference from the many other proteins, peptides, etc. present in the sample. The most effective way to carry out this type of testing is a ligand-binding assay. Specific monoclonal antibodies (mAbs) will be developed against the analyte and evaluated for their affinity to the analyte of interest to determine which antibody candidates will work best in the assay (Leary et al., 2013).

Below, as seen in Figure 1, is an example of a type of ligand-binding assay format known as a sandwich ELISA. To measure the serum concentration of a biotherapeutic, the drug can be specifically 'captured' from the serum by an antibody that is specific to a unique epitope on the drug itself. The capture antibody is coated to the well of a 96-well plate, where it will bind any drug present in a properly diluted serum sample. After the capture step, the unbound, excess serum and other unwanted proteins present can be washed away, and a second enzyme conjugated antibody is added for detection. The detection antibody is selected to be specific against a second, unique epitope on the drug and allows one to accurately detect the amount of drug captured in a well after a second wash to remove any excess reagent. Upon addition of an enzyme substrate to 'develop' the wells–an enzymatic process which produces an amount of color in the well that is proportional to the amount of drug detected–the plate can be read at the proper wavelength and the optical response in each well can be compared to a known standard dilution to determine sample concentration (Wakankar, 2011).

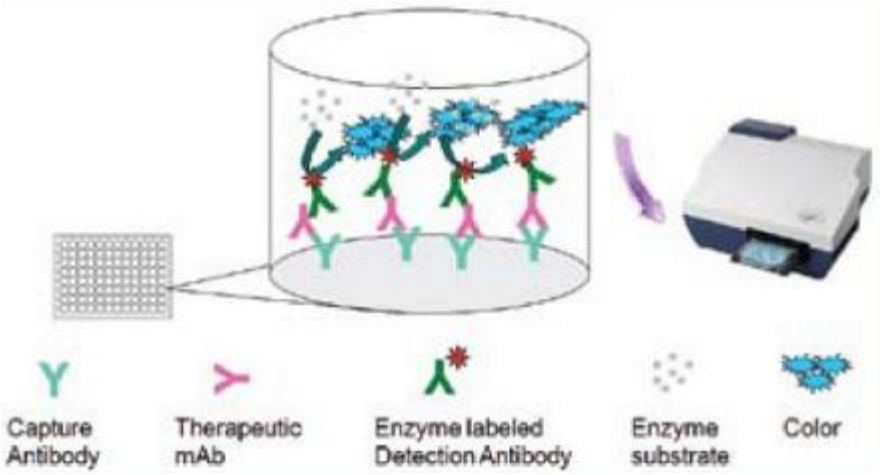


Figure 1: Graphical depiction of an ELISA assay performed in a sandwich format (Wang, 2010).

The term pharmacodynamics (pD) refers to the relationship between drug concentration at the site of action and the resulting effect, including the course of time and the intensity of both therapeutic and adverse effects. In general, the effect of a drug present at the site of action is determined by the level to which that drug binds with a receptor. These receptors may signal cell-uptake, initiate downstream cell-signaling, or have any number of other effects on the body. The concentration of a drug at the site of the intended receptor typically determines the intensity of that drug's effect. The level of effect is also modulated by the density of receptors on the target cell type, the mechanism of signal transmission in the cell, and any related regulatory factors that govern downstream effects.

An important factor that can affect the pharmacokinetics, and thus the pharmacodynamics, of a biotherapeutic treatment is the immune system of the patient being dosed. If a treatment causes an immune reaction in the patient (or, in other words, the treatment is immunogenic in the patient), the patient may begin to manufacture specific anti-drug antibodies (ADAs) to attack and clear the offending substance from the blood. This unwanted

immune response can reduce the length of time a drug remains functional in the patient's body and thus, reduce its therapeutic value or even neutralize the treatment altogether. Although every patient has the potential to react differently to a given treatment, biotherapeutics can often be more immunogenic than their small-molecule counterparts. This factor makes immunogenicity studies especially important for biotherapeutics (Hock et al., 2015).

In order to study immunogenicity and monitor patients for this type of deleterious immune reaction, a type of ligand-binding assay known as an anti-drug antibody assay can be employed. In an ADA assay (Figure 2), similar to the generic sandwich ELISA format previously discussed, the drug (a monoclonal antibody for example) can be conjugated to biotin and either 'pulled-down' onto a streptavidin-coated plate, or otherwise bound to the wells of a standard 96-well plate. Patient serum can then be added to the well and incubated, which will result in the binding of any anti-drug antibody present in the serum to the drug captured in that well. The uncaptured excess is subsequently washed away and a second drug conjugate (conjugated to a detection mechanism) is then added. The second conjugate becomes bound in a sandwich conformation to the patient's ADAs and can then be detected in a number of ways, depending on the detection mechanism used for the assay platform. If a patient is found to be positive for ADAs, further testing is often run to determine whether the antibodies have a neutralizing effect on the drug treatment, or if they merely bind to it without causing any discernible changes (Stephan et al., 2011).

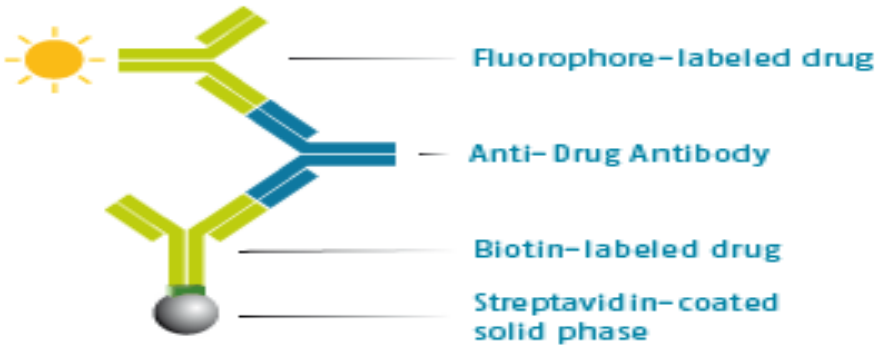


Figure 2: Generic ADA assay format.

(Retrieved from: http://www.gyros.com/user-zone-2/assay-development-guidelines-ada/).

The ligand-binding assay is the method of choice to quantify levels of a target analyte in a complex protein mixture or 'matrix.' This type of assay has only grown in prominence as drug companies increasingly shift towards the development and manufacture of biotherapeutics. Ligand-binding reagents (critical reagents), when used in an appropriate assay format, are the most effective method to uniquely bind and identify an analyte of interest amongst a multitude of other components in a complex matrix like human serum. This type of specific interaction allows a researcher to detect the presence of the drug or ADA in patient serum without interference (Pollard, 2010; Wakankar et al., 2011).

Antibody Development and Purification:

Monoclonal antibodies are generally produced in cell culture by fusing myeloma cells to either mouse spleen cells or rabbit B-cells, which have been immunized with an antigen of interest. Upon immunization, these hybrid myeloma cells will produce identical copies of a single antibody against a specific epitope on the analyte that was used to immunize the animal.

In general, when developing mAbs for use as critical reagents in an LBA, several mAb candidates will be grown in culture and evaluated for use in the assay. The antibodies themselves are excreted into the supernatant by the cultured cells and require subsequent purification and buffer exchange prior to further use in assay development.

For mAbs (IgG), the easiest and most effective method for purification is a Protein A/Protein G column. Protein A and Protein G are immunoglobulin-binding proteins which can be recombinantly expressed and used to purify, immobilize and detect immunoglobulins. Each of the immunoglobulin-binding proteins has a different binding profile with respect to the portion of the antibody that is recognized and the isotype that it will bind. These proteins, when bound in a purification column, can be used to bind and extract all of the IgG present in a cell culture supernatant (or animal serum), thus purifying out the IgG portion (the mAbs themselves, in this case) from the unwanted culture media and metabolic waste that is also present in the supernatant. Upon elution from the column, only the desired IgG mAbs will be present in the elution buffer used. A final buffer exchange step can then be performed to facilitate stable storage (Wikipedia Contributors, 2013).

Polyclonal antibodies (pAbs), obtained from the serum of immunized animals, require a further purification step. After collecting the serum of an animal that has been inoculated against the target of interest, the polyclonal IgG in the serum can be similarly purified using a Protein A/Protein G column. Once this step is complete, all of the IgG present in the animal, or 'whole IgG' will have been collected from the serum, including some portion that is specific to the target of interest, which must then be purified from the remaining, non-specific IgG population. In order to select out only those IgGs specific to the desired target, an affinity purification step must be performed.

Small-scale affinity purification, such as that done for the production of critical reagents, is often carried out using a commercially available column resin, which can be coupled to the target of interest. The whole IgG portion, collected from the prior Protein A/G purification step, is then passed over the coupled affinity resin and the specific pAbs will be bound to the target; this allows the unbound, non-specific portion to be easily washed away. After washing the unbound antibodies from the column with several buffer rinses, the specific polyclonal IgG is then eluted using a low pH solution (pH ~2.4) and then immediately pH neutralized upon collection. The collected antibodies can then be buffer exchanged and stored for later use (Filntisi et al., 2014).

Chemistry of Protein Conjugation:

The process of protein conjugation involves the linking of proteins or other biological molecules to conjugate molecules through a chemical reaction. This includes the conjugation of antibodies, enzymes, peptides, or other biologically active molecules to any type of molecule that adds a desirable property. These molecules may include radioactive tags, toxins, enzymes or fluorescent compounds, among many others. The most common methods used to carry out such protein conjugations involve amine chemistry and can easily be performed by a single analyst in a lab (Breen et al., 2016; Sesay, 2003).

One of the most common reactive groups found on proteins is the aliphatic ε-amine of the amino acid group lysine. Nearly all antibodies have lysine residues in abundance, and it is therefore, a useful target when performing conjugations. The amines in lysine become reasonably nucleophilic above pH 8.0 and can react easily with a variety of reagents to form stable bonds. These coupling reactions may be performed at multiple challenge ratios (or

differing conjugate dilutions) in an attempt to induce the proper level of conjugation (Sesay, 2003).

Common antibody-amine conjugation reaction:

Antibody-NH_2 + Z-B →

Antibody-NHB + Z-H (1)

Other reactive amine groups found on proteins are the α-amino groups of *N*-terminal amino acids. The α-amino groups are less basic than lysine residues and are reactive at a lower pH (approximately 7.0). Performing a conjugation reaction at a pH of 10, for example, will render these *N*-terminal sites non-reactive. Conversely, carrying out the reaction at a lower pH will allow the *N*-terminal amino groups to be to be targeted. Since either *N*-terminal amines or lysine residues are almost always present, and because these sites can be conjugated with relative ease, these amines provide the most commonly employed method of protein conjugation (Filntisi et al., 2014; Sesay, 2003).

In general, when performing protein conjugation reactions of this type, one must be mindful of the statistical and somewhat random nature of such reactions. Thought must be given to the potential effect the conjugation reaction itself can have on the final conjugated product. The exact sites of conjugation cannot be strictly controlled and may vary from one conjugate molecule to another. Whether each potential conjugation site is chemically "equal" is unknown, as some sites may affect binding affinity, potential epitopic regions, or other chemical properties of a mAb where alternative sites may not.

In such reactions, the inherent uncertainty very often leads to a heterogeneous, polydispersed population of conjugates, some with different conjugation sites, degrees of

labeling, and possibly, a somewhat altered set of chemical properties. The conjugation process has the potential to perturb the conjugated protein's tertiary structure in subtle ways, some of which may induce changes to the hydrophobicity, charge or level of aggregation (see Figure 4 – Panel A). When preparing critical reagents for an important LBA, it is helpful to keep these factors in mind. Once reagent conjugation has been completed, it is always good practice to measure the binding affinity of the final conjugate against that of the unconjugated Ab to ensure proper binding was retained (Filntisi et al., 2014, Wakankar et al., 2011).

Area of Specific Interest:

Custom-made, critical reagents designed for use in testing are usually produced in relatively small batches, either in-house or through a contract partner. Reagents of this type are required to perform ligand-binding assays (LBA), such as anti-drug antibody (ADA) assays, in the modern lab. The term critical reagent describes an essential component of a ligand-binding assay whose unique characteristics directly impact assay performance. Throughout biotherapeutic drug development and the various clinical phases, LBA and ADA assays are run to assess and monitor several crucial parameters of a given biotherapeutic treatment, such as pK, pD and immunogenicity.

Ligand-binding assays are the primary methods used to quantify biotherapeutics, biomarkers and anti-drug antibodies to support biotherapeutics development. Ligand-binding assays take advantage of an antibody's or receptors unique specificity for its target to measure analytes, even in the presence of a complex matrix or background. Researchers may use an antibody that was raised against a specific epitope, or set of epitopes, on the analyte of interest

(e.g., the biotherapeutic drug itself, the drug's target, etc.) (O'Hara et al., 2012; Wakankar, 2011). The specificity of the antibodies employed in the assay allows one to measure the analyte effectively, even when it is mixed within a matrix of hundreds or thousands of other proteins (as it would be in a patient's serum), with very little interference. These Abs are referred to as critical reagents because ligand-binding assays cannot be performed without them. Thus, the continued specificity, stability and overall quality of these critical reagents are directly related to the accuracy and precision of the assay and the quality of any data collected from it. Therefore, assays of this type necessitate a thorough and accurate physiochemical characterization of any critical reagent produced for testing to ensure a high degree of quality (O'Hara et al., 2013).

The production of critical reagents often involves the conjugation of a source protein to either a capture (e.g., biotin) or a detection mechanism (e.g., a fluorescent label or dye conjugate) for functional use in an assay. The source protein could be a specific monoclonal antibody cultured in a lab, a mass produced biotherapeutic like a mAb or enzyme, like many of the biotech drugs currently on the market, or a polyclonal antibody that has been purified from the serum of an animal previously inoculated against the target of interest. Animal pAbs, in their native state, are often utilized as positive controls in ligand-binding assays (O'Hara et al., 2013).

As a biochemical process, the statistical nature of protein conjugation often introduces polydispersity and other forms of physical heterogeneity into the conjugated population. Polydispersity, or heterodispersity, is a term that describes the distribution of particles of varied sizes which are *dispersed* in a colloidal solution. Polydispersity can proceed naturally over time or occur more rapidly due to changes in pH, perturbation of the protein's tertiary structure by the conjugation process, or by induced hydrophobic or ionic forces related to the conjugation. Some of the proteins in the solution may aggregate, leading to a mixed population of monomeric,

dimeric, trimeric, etc. aggregations of protein subunits (Frka-Petesic et al., 2016). This raises a question for those tasked with producing high quality critical reagents: What effect does the polydispersity of a source protein have when used as the basis for the production of a conjugated critical reagent? Could this potentially affect the degree to which the source protein is tagged by the intended conjugate, also known as degree of labeling (DOL)? What affect would such phenomena have on the results of an LBA for which the critical reagent is intended?

In practice, critical reagents are usually found to be aggregated to some degree, and the DOL of the dispersed populations may vary. Depending on the nature of the purification and conjugation procedure used, the level of aggregation and polydispersity can differ greatly from one protein to another, taking a wide range of values. The working theory regarding the effect that polydispersity has on the product of a conjugation reaction presented in this thesis is as follows:

> When conjugating a protein to a detection mechanism, or any other conjugate of interest, the reaction, in solution, can be visualized as occurring in a vast, empty space. In such a scenario, each particle in the reaction is very dilute relative to the total volume of the reaction. Therefore, each protein particle in the reaction has a similar, average number of chemical interactions with the conjugate molecules in the reaction, regardless of the protein particle's size or number of subunits. When comparing the size of small protein particles (≤150,000 KDa), such as a single enzyme or antibody, the difference in "size" between a single and multi subunit "aggregate" is quite negligible relative to the total volume of the conjugation reaction. The number of molecular interactions a particle will have during the conjugation reaction is very unlikely to be affected by the number of subunits it contains.

However, significant levels of aggregation could absolutely affect the degree of labeling in a polydispersed conjugate population precisely because all of the particles in the reaction are likely to average the same number of molecular interactions during the reaction. If the monomeric portion of the dispersed protein population receives one label on average, the aggregated portion will have the same number of labels, regardless of how many subunits the particle contains. However, because the particles do not contain the same number of subunits, the degree of labeling may differ significantly. This is because the amount of protein present is inversely related the degree of labeling; as the protein concentration increases, the degree of labeling decreases.

In Figure 3, a protein-conjugate sample under analysis was separated by size exclusion chromatography (SEC) on a Waters Alliance HPLC instrument. In SEC analysis, the intact, non-denatured protein sample is passed through a column packed with specialized beads under high pressure. The column is designed to retain smaller particles for a longer period of time than larger particles, in order to separate them by their size vs. their retention time, as depicted by the x-axis. The largest particles in the solution take a shorter path through the column packing material and are eluted first (farther to the left on the x-axis), with a lower retention time, while the smaller particles take longer paths through the column packing material and are eluted later, with increasing retention times according to a decreasing size gradient. After separation by size, the separated sample passes through a series of detectors and can detected simultaneously for absorbance at 280 nm for protein content and aggregation, and also at the proper excitation and emission wavelengths for fluorescence response in accordance with the fluorescent tag used to

produce the conjugate being assayed. In Figure 3, the two chromatograms, one received from each detector, are overlayed and normalized to the y-axis for comparison.

All SEC data is interpreted in a relative manner according to the percent area under each peak. Shown by the black-colored A280 nm trace, the sample in Figure 3 has a large aggregated, high molecular weight (HMW) portion relative to the amount of smaller constituents in the overall polydispersed size population. However, when comparing the information received from each detector, the aggregated portion of the reagent appears to have a much lower fluorescence response per amount of protein present (according to the blue-colored trace), leading to the inference that this HMW population may have lower DOL in comparison to the monomeric or dimeric portions of the reagent. Thus, depending on the severity of the aggregation and the potential disparity in DOL of the polydispersed populations, this phenomenon could potentially have an effect on the sensitivity or precision of any ligand-binding assay in which this lot of reagent is to be used. For example, assume our critical reagent contains an aggregated and less-labeled, dimeric portion, which retains its biological activity in the assay. That dimeric portion of the critical reagent can bind two-fold the number of target ligands as the monomeric portion, yet it will provide only an equal response upon detection, leading to an underestimation of the concentration of our target and compromising the accuracy of our results.

Chapter II

Materials and Methods

For the purposes of this thesis, two conjugates were identified through preliminary work, which were thought to be good candidates for studying the effects of protein aggregation on the DOL of polydispersed protein conjugate populations. Due to restraints around corporate privacy, these conjugates can only be referred to as ERT1-AF594 and ERT2-AF488, meaning enzyme replacement therapy 1 conjugated to Alexa Fluor 594 and enzyme replacement therapy 2 conjugated to Alexa Fluor 488 respectively. These two enzyme replacement therapies (ERTs) are products developed by the Sanofi Genzyme Company and are conjugated to various fluorescent reporters for the purpose of study in clinical assays, such as assays for cell uptake and neutralizing anti-drug antibody. A lot of each of these ERT conjugates was specifically prepared for use in this study.

Conjugation of ERT1-AF594:

Two 20 mg vials of ERT1 drug substance were reconstituted to a concentration of 10 mg/mL with DI water. The reconstituted ERT1 was then desalted using a 10 mL, 7K MWC Zeba Spin Column (Thermo Science) and buffer exchanged into a 0.1 M Sodium Bicarbonate solution (pH 8.3) using a second, 10 mL, 7K MWC Zeba Spin Column that was pre-washed 4x with 0.1 M Sodium Bicarbonate solution (pH 8.3). The desalted and buffer exchanged ERT1

was then adjusted to 5 mg/mL using the same bicarbonate buffer as above and stored at 2-8°C while the Alexa Fluor 594 conjugate material was prepared. All spins were done at 1000 RCF for 2 minutes at ambient temperature.

An appropriate amount of 5 mg/mL Alexa Fluor 594 (AF594), a commercial reagent sold by Life Technologies, was prepared to perform the conjugation at molar ratio of 20 (20 mol dye to 1 mol protein). Two 5 mg AF 594 aliquots were reconstituted with 500 uL DMSO per aliquot, mixed well and protected from light prior to conjugation.

In a dark area, the reconstituted AF594 was added dropwise to the ERT1 in 0.1 M Sodium Bicarbonate solution (pH 8.3) while stirring vigorously. The conjugate mixture was then protected from light and incubated at 2-8°C for 1 hour on a mixer.

After stirring at 2-8°C for an hour, the final conjugate was immediately desalted into 1x PBS (pH 7.2) on an AKTA Explorer instrument using a 5K MWC HiPrep 26/10 Desalting Column (GE Healthcare) flowing at 5.0 mL/min to remove any free, unconjugated dye molecules and to buffer exchange the ERT1-AF594 into 1x PBS for storage. (Refer to Figure 5)

Conjugation of ERT2-AF488:

Six vials of ERT2 drug substance were obtained and concentrated using 6 9K MWC, 7mL Pierce Protein Concentrators (Thermo Science) that had been prewashed with 6 mL DI water and spun at 2000 RCF for 10 minutes. Once the ERT2 was added to each concentrator, they were spun at 2500 RCF for 25 minutes at ambient temperature. After removing the concentrated ERT2 from each concentrator, the concentrators were loaded with 60 uL DI water

and allowed to sit at ambient temperature for 10 minutes, after which the 60 uL of water was removed and added to the concentrated ERT2 samples and stored at 2-8° C. A BCA assay was then performed to determine the final protein concentration of the concentrated ERT2 samples.

Each ERT2 sample was then diluted to a concentration of 5 mg/mL with DI water based on the results of the previous BCA assay. Then, with the ERT2 samples on ice, the pH of each sample was brought to a pH of 9.0 through addition of a 1M Sodium Bicarbonate solution (pH 9.4) and stored at 2-8°C.

An appropriate amount of 10 mg/mL Alexa Fluor 488 (AF488), a commercial reagent sold by Life Technologies, was prepared to perform the conjugation at molar ratio of 25 (25 mol dye to 1 mol protein). The AF488 aliquots were reconstituted with 500 uL DMSO per aliquot, mixed well and protected from light prior to conjugation.

In a dark area, the reconstituted AF488 was added dropwise to the ERT2 at pH 9.0 while stirring vigorously. The conjugate mixture was then protected from light and incubated at 2-8°C for 1 hour on a mixer.

After stirring at 2-8°C for an hour, the final conjugate was immediately added to a 10,000 MWC Slide-A-Lyzer™ dialysis cassette (Thermo Science) and dialyzed into 4L of pre-chilled 1x PBS at 2-8°C. The pre-chilled 1x PBS was subsequently changed 5x with fresh, pre-chilled 1x PBS over a 60 hour period, with at least 4 hours between each buffer change. After completing the 5 rounds of dialysis, the ERT2-AF488 was harvested and protected from light for storage.

Fractionation of ERT1-AF594 and ERT2-AF488 on AKTA Explorer:

To study the individual, poly dispersed populations in each prepared conjugate, each ERT conjugate was fractionated into 0.5 mL fractions using an AKTA Explorer instrument (GE Healthcare), and collected using an associated fraction collector and 3 generic 96-deepwell plates. A GE HiPrep 26/60 Sephacryl™ S-2000 semi-analytical SEC column (GE Healthcare) was used to fractionate the samples, flowing at 5 mL/min in 100 mM Sodium Phosphate, 200 mM L-Arginine buffer (pH 6.8). (Refer to Figures 6 and 7)

Determination of Degree of Labeling:

A NanoDrop 2000 instrument was used to determine the final protein concentration, dye absorbance and DOL of selected fractions from the previous experiment. The NanoDrop 2000 instrument functions almost identically to a spectrophotometer, but requires one to use much less sample per measurement (2 uL). Select fractions of each ERT conjugate were measured on the instrument to determine the protein concentration and dye absorbance for the given fractions in order to specifically determine the DOL for each. (Refer to Tables 2 and 3)

Confirmation of Proper Separation and Fractionation by Analytical Size Exclusion HPLC:

The final step of the study was to confirm through analytical SEC that the fractions collected had, in fact, been properly separated by size and that the polydispersed populations had indeed been isolated or enriched in the fractions tested. In other words, this technique was used to determine which size populations had been captured or enriched in each fraction of interest. (Refer to Figures 7-10)

The SEC testing was performed on a Waters Alliance HPLC system using a Waters 300 mm Xbridge BEH200 (or BEH450) SEC column using a 100 mM Sodium Phosphate, 200 mM L-Arginine mobile phase (pH 6.8). A protein load of 20 ug per injection was targeted based on the protein concentration data collected for each fraction in the previous study using the NanoDrop 2000. The samples were run at a flow rate of 1 mL/min for 20 minutes and detected at 280 nm.

Chapter III

Results:

Preliminary Data:

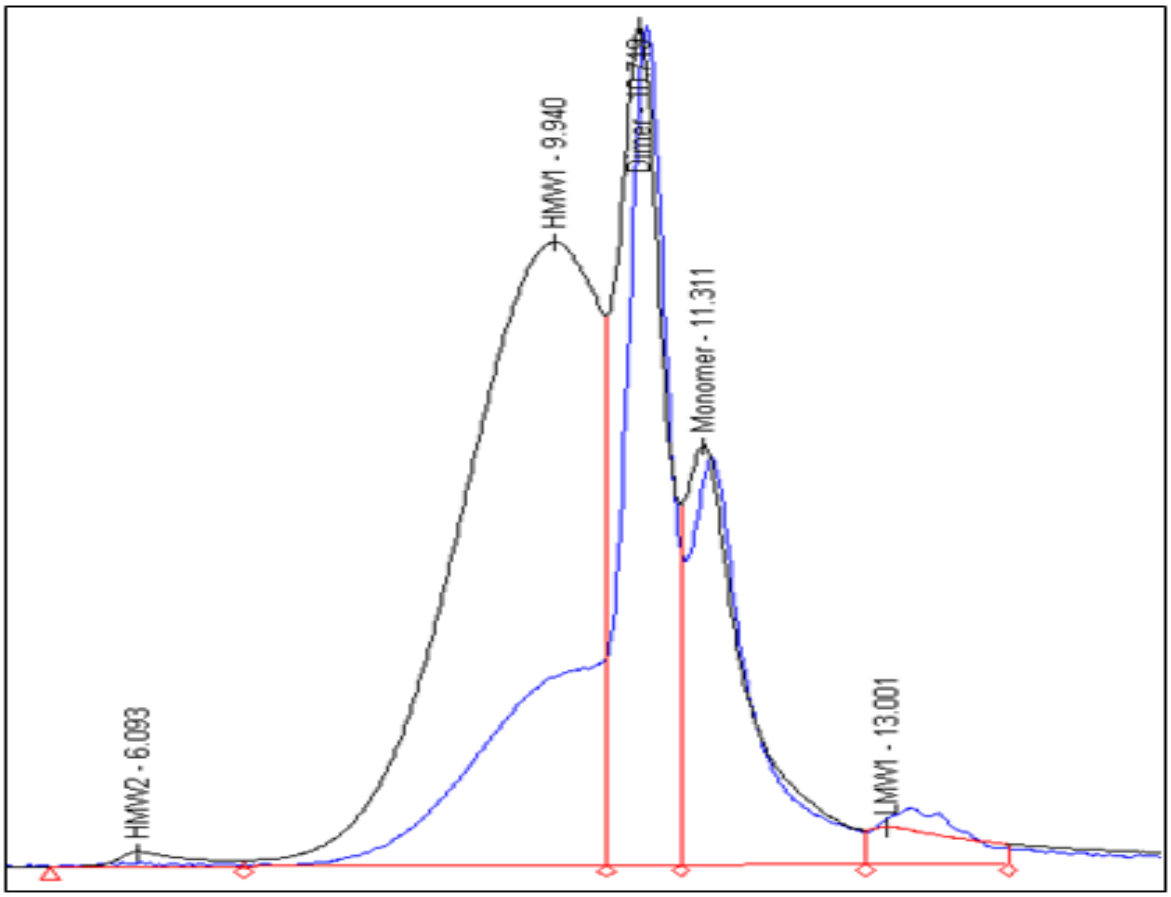


Figure 3: Here we see an overlay of SEC data showing the 280 nm trace and the fluorescence trace for a fluorescent dye conjugate. The 280 nm profile is shown in black and the fluorescence profile is superimposed in blue. Overlaying the 280 nm and fluorescence data allows us to clearly visualize the significant discrepancy between the levels of fluorescence signal given off by the different polydispersed populations in the conjugate sample. The more aggregated portion of the dispersed population appears to be considerably less labeled than the less aggregated portion of the sample.

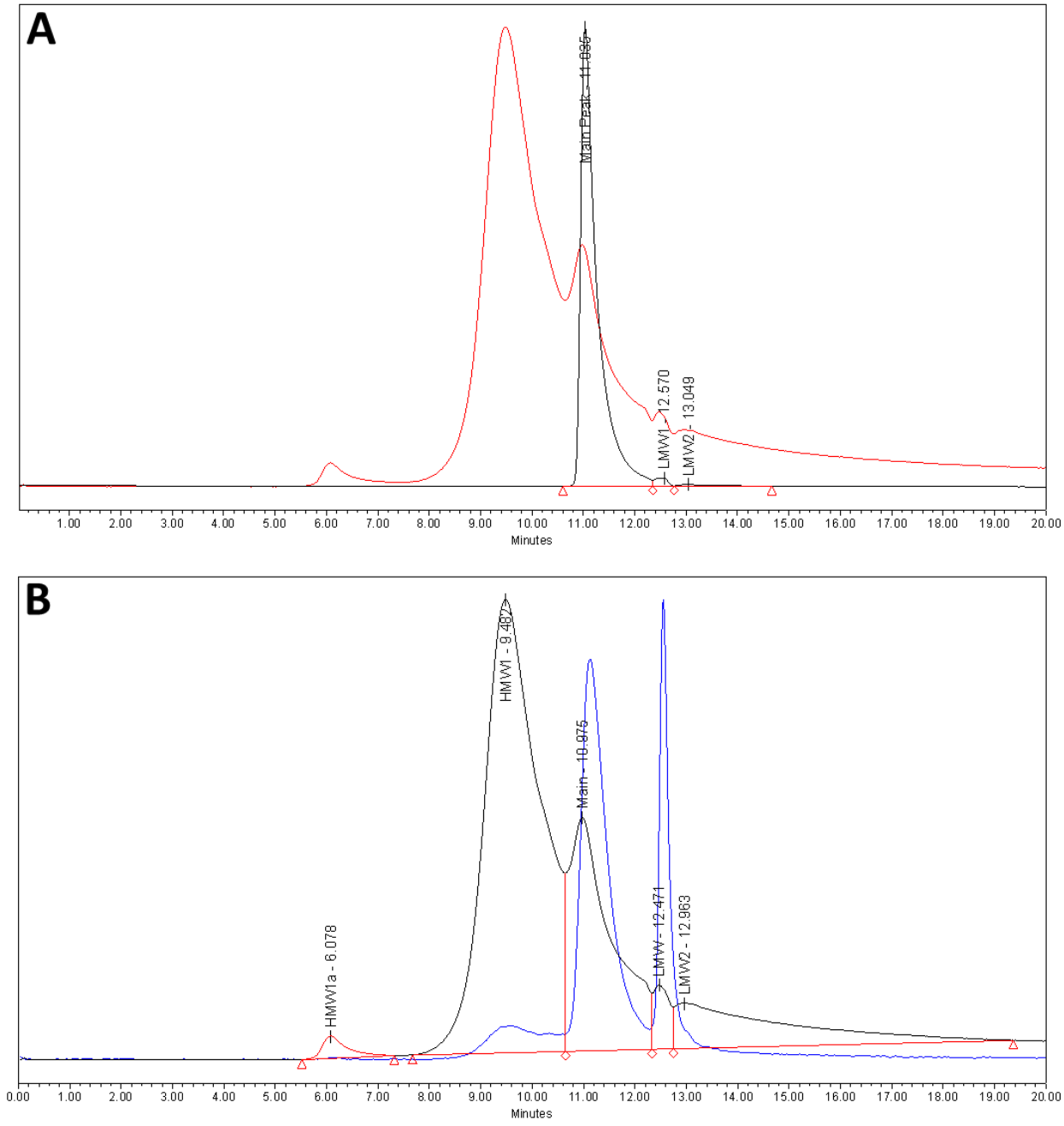


Figure 4A: Analytical SEC of ERT2 drug product (black) and ERT2-AF488 conjugate (red), both detected at 280 nm. 20 ug of each protein was separated on a Waters 300mm Xbridge BEH450 SEC column using 100 mM Sodium Phosphate, pH 6.8 mobile phase. Quantification of peak areas is summarized in Table 1. Note the significant increase in aggregation from before and after the conjugation reaction. NOTE: The overlay of the initial, unlabeled ERT2 drug product, superimposed against the final product of the conjugation reaction, is a very effective way to visualize how severely the conjugation reaction can affect the polydispersity of the sample. The initial drug product (shown in

black in panel A) is a single monomeric peak with a very small amount of LMW species. However, the final conjugation product (shown in red in panel A) shows significantly increased levels of aggregation and polydispersity, the apparent result of some aspect of the conjugation reaction itself.

Figure 4B: Analytical SEC of ERT2-AF488 dye conjugate. Due to a path length of several centimeters between detectors, the retention time of all Ex495/Em519 fluorescence peaks (blue lines) is very slightly delayed relative to the corresponding 280 nm peaks (black lines). The y-axis is normalized such that both fluorescence and 280 nm channels are displayed on the same scale. Proteins were separated on a Waters 300mm Xbridge BEH450 SEC column using 100 mM Sodium Phosphate, pH 6.8 mobile phase (20 ug per injection for A280 detection; 1.0 ug per injection for fluorescence detection). Quantification of peak areas is summarized in Table 1.

	% of Total Peak Area					
	A280 (total protein)		***Em519 (fluor. Protein)***		***Fluor/A280 Ratio***	
Sample	***Main Peak***	***HMW Peaks***	***Main Peak***	***HMW Peaks***	***Main Peak***	***HMW Peaks***
ERT2 drug product	97.84	N/A	N/A	N/A	N/A	N/A
ERT2-AF488 (corrected for free dye peak)	23.73	61.69	84.84	15.16	3.575	0.246

Table 1: Summary of analytical SEC data generated for ERT2 drug product and ERT2-AF488 dye conjugate protein. The percent of total peak area is reported for both A280 and Ex495/Em519 channels, and the fluorescence/A280 ratio is calculated from the corresponding peak area percentages. Data is shown with a correction for the free dye associated signal. The free dye peak in the sample accounts for ~27.5% of the total fluorescence area. The chromatographic data is shown in Figure 4B.

NOTE: A further analysis of the SEC data in Figure 4B was performed in which the ratio of peak area percentages (fluorescence vs. A280) was examined for the main and HMW peaks of the ERT2-AF488 conjugate (see Table 1). This ratio should have a value close to 1.0 provided that dye-to-protein incorporation is approximately equal in both species. In our analysis, however, the ratio was observed to be far >1 for the main peak (~3.58) and far <1 for the HMW peak (~0.25) fractions after correcting for the free dye-associated signal. We conclude that the degree of labeling for the main peak population is substantially higher than that of the HMW populations.

Conjugation Data (Desalting and Buffer Exchange of ERT1-AF594):

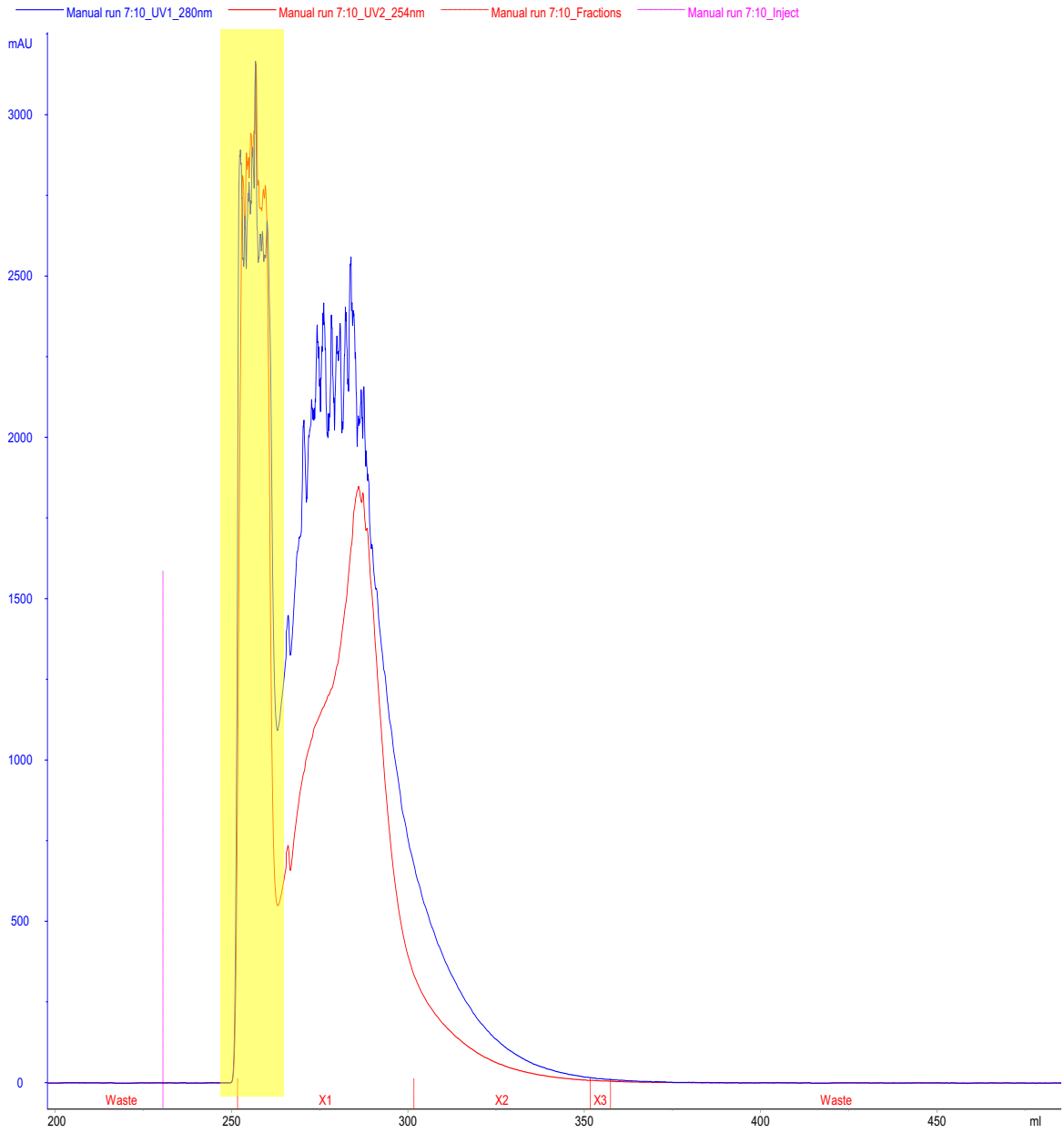


Figure 5: Chromatogram from the desalting and buffer exchange steps from the ERT1-AF594 conjugation protocol. In order to remove any remaining free dye from the final conjugate product, the sample was run through a 5K MWC HiPrep 26/10 Desalting Column (GE Healthcare) flowing at 5.0 mL/min, and was buffer exchanged into 1X PBS, pH 7.2. The collected portion of the sample is highlighted in the yellow area above. The remainder of the sample (everything outside of the yellow area) was not collected, as this portion contains mostly free, unreacted dye. This Figure is simply intended to help the reader better understand what is being done during the buffer exchange step, how the data is displayed and to aid in visualizing which portions of the sample are collected and which portions are considered to be waste.

Fractionation Data:

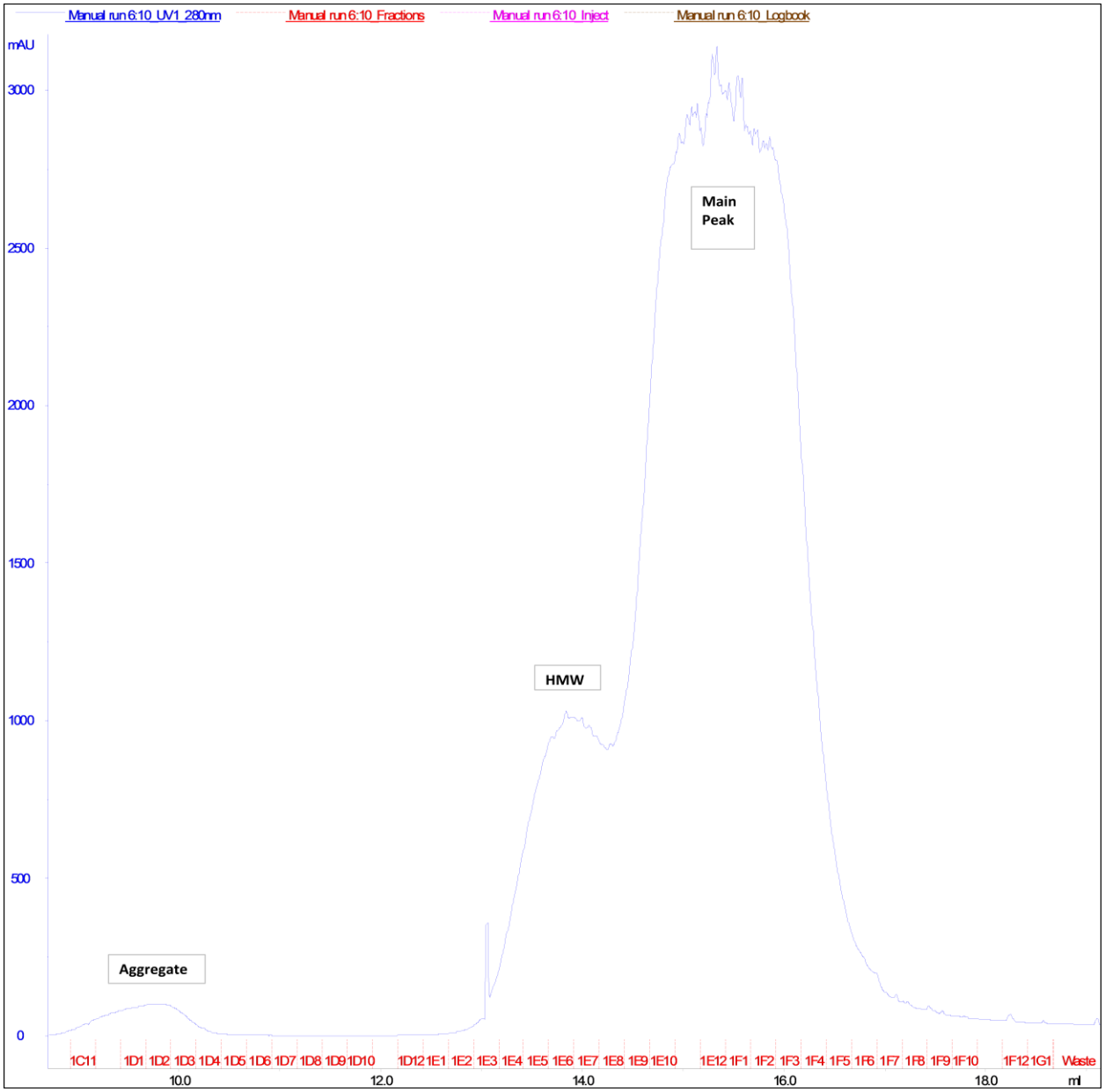


Figure 6: Chromatogram of 280 nm signal (shown in blue) and the fractions collected during ERT1-AF594 fractionation (shown below the chromatogram in red). ERT1-AF594 conjugate was fractionated into 0.5 mL fractions using an AKTA Explorer instrument (GE Healthcare), and collected using an associated fraction collector and 3 generic 96-deepwell plates. A GE HiPrep 26/60 Sephacryl™ S-2000 semi-analytical SEC column (GE Healthcare) was used to fractionate the samples, flowing at 5 mL/min in 100 mM Sodium Phosphate, 200 mM L-Arginine buffer (pH 6.8).

For the ERT1-AF594 sample, it was determined that the fractions of interest would be (listed from left [larger] to right [smaller] by retention time): 1D2, 1E5, 1E6, 1E7, 1E10, 1F1 and 1F3.

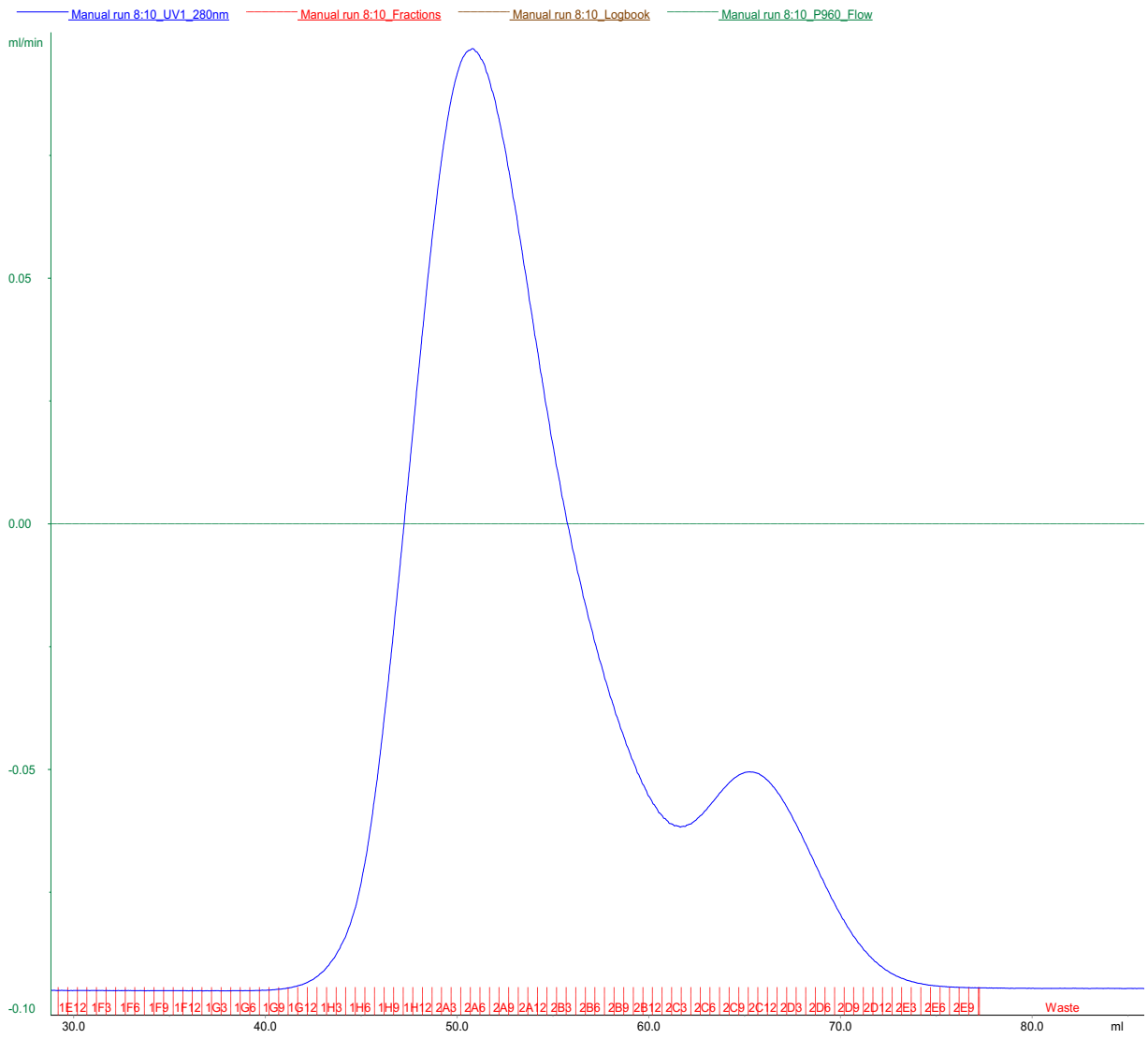


Figure 7: Chromatogram of 280 nm signal (shown in blue) and fractions collected during ERT2-AF488 fractionation (shown below the chromatogram in red). ERT2-AF488 conjugate was fractionated into 0.5 mL fractions using an AKTA Explorer instrument (GE Healthcare), and collected using an associated fraction collector and 3 generic 96-deepwell plates. A GE HiPrep 26/60 Sephacryl™ S-2000 semi-analytical SEC column (GE Healthcare) was used to fractionate the samples, flowing at 5 mL/min in 100 mM Sodium Phosphate, 200 mM L-Arginine buffer (pH 6.8). Referring to the overlay in Figure 4 – Panel A, we see it is the smaller of the two peaks that represents the non-aggregated portion of ERT2.

For the ERT2-AF488 sample, it was determined that the fractions of interest would be (listed from left [larger] to right [smaller] by retention time): 1H9, 1H12, 2A6, 2A9, 2B3, 2B6, 2C6, 2C9, and 2C12.

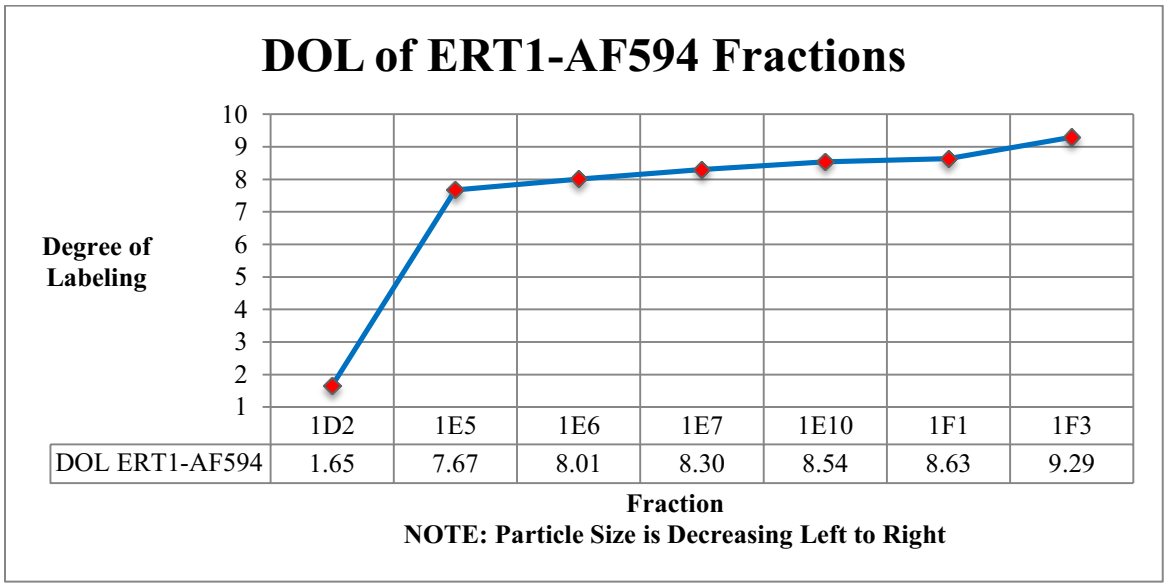
Data from DOL Determination for ERT1-AF594:

Fraction:	Corrected Protein Concentration by A280 (mg/mL):	Corrected Dye Absorbance by A590 (mg/mL)	DOL	Ratio of DOL vs. Max DOL	% DOL of Max DOL
Unfractionated Whole	1.325	14.1219	7.94	N/A	N/A
1D2	0.03	0.067	1.65	0.177718408	17.77%
1E5	0.11	1.133	7.67	0.82554101	82.55%
1E6	0.14	1.505	8.01	0.861608336	86.16%
1E7	0.1575	1.755	8.30	0.893095683	89.31%
1E10	0.3067	3.516	8.54	0.918832859	91.88%
1F1	0.415	4.811	8.63	0.929156111	92.92%
1F3	0.3	3.743	9.29	1	100.00%

Table 2: Data and calculated DOL for ERT1-AF594 conjugate fractions of interest. DOL for ERT1-AF594 was determined as follows:

DOL = [A_{590}] ERT1-AF594 x (Mol. Weight of ERT1) / [ERT1-AF594] mg/mL / ε of AF594

Mol. Weight of ERT1 = 70,000; the absorbance coefficient (ε) of AF594 = 94,000



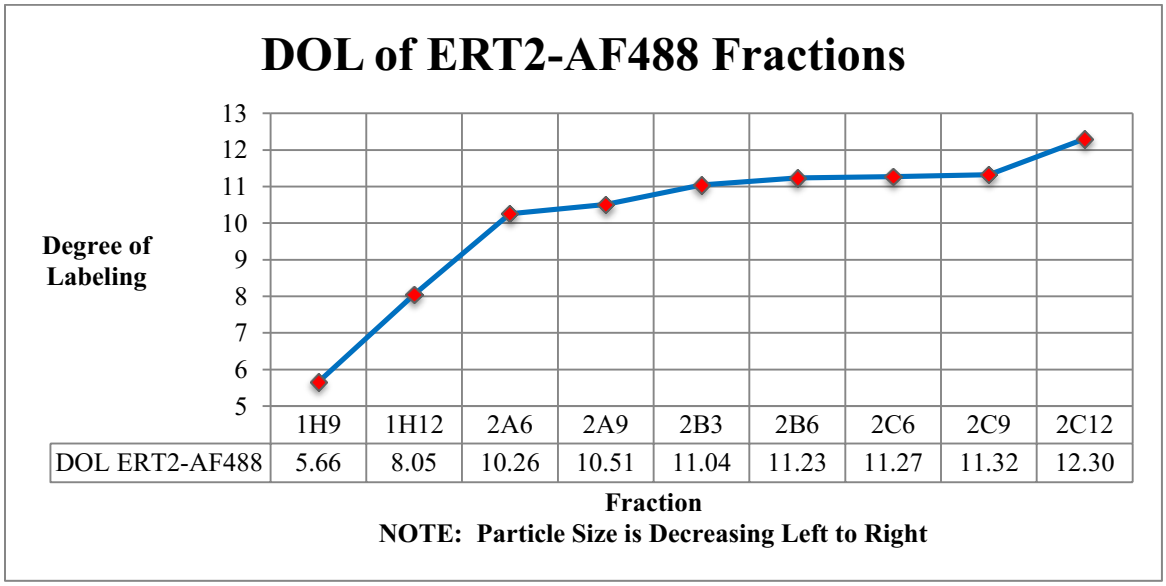
Data from DOL Determination for ERT2-AF488:

Fraction:	Corrected Protein Concentration by A280 (mg/mL):	Corrected Dye Absorbance by A495 (mg/mL)	DOL	Ratio of DOL vs. Max DOL	% DOL of Max DOL
Unfractionated Whole	1.645	12.54	8.91	N/A	N/A
1H9	0.08	0.387	5.66	0.45980198	45.98%
1H12	0.22	1.515	8.05	0.654545455	65.45%
2A6	0.535	4.694	10.26	0.833948367	83.39%
2A9	0.58	5.2135	10.51	0.854380335	85.44%
2B3	0.42	3.9665	11.04	0.897652051	89.77%
2B6	0.315	3.027	11.23	0.913380481	91.34%
2C6	0.105	1.012	11.27	0.916096181	91.61%
2C9	0.115	1.114	11.32	0.920740422	92.07%
2C12	0.12	1.2625	12.30	1	100.00%

Table 3: Data and calculated DOL for ERT2-AF488 conjugate fractions of interest. DOL for ERT2-AF488 was determined as follows:

DOL = [A_{495}] ERT2-AF488 x (Mol. Weight of ERT2) / [ERT2-AF488] mg/mL / ε of AF488

Mol. Weight of ERT1 = 83,000; the absorbance coefficient (ε) of AF488 = 71,000



Data from Analytical SEC:

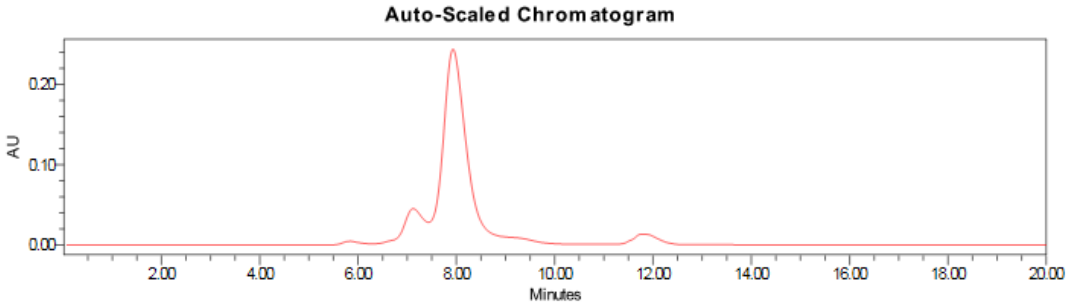


Figure 8: Chromatogram of the unfractionated ERT1-AF594 conjugate run by analytical SEC and detected at 280 nm. Analytical SEC for ERT1-AF594 was performed on a Waters Alliance HPLC system, using a Waters 300 mm Xbridge BEH200 SEC column, and a 100 mM Sodium Phosphate, 200 mM L-Arginine mobile phase (pH 6.8). A protein load of 20 ug per injection was targeted, running at a flow rate of 1 mL/min for 20 minutes and detected at wavelength of 280 nm.

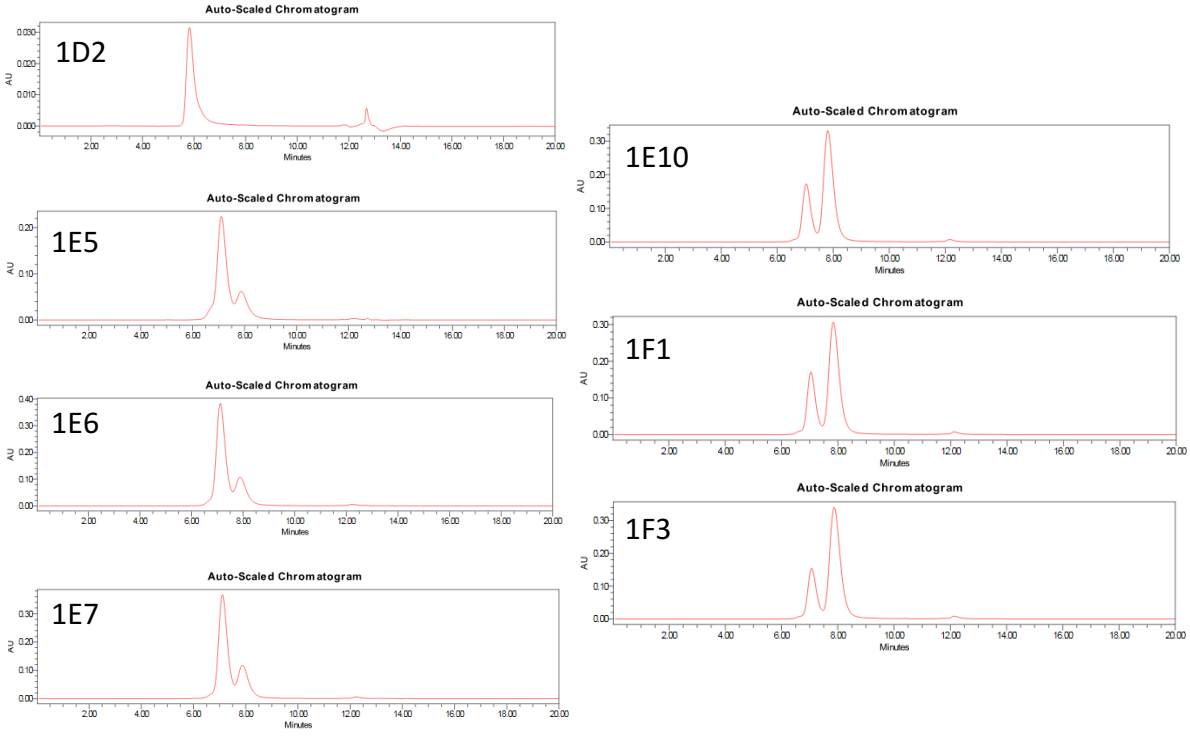


Figure 9: Chromatograms of each fraction of interest for ERT1-AF594 run by analytical SEC. Analytical SEC for ERT1-AF594 was performed on a Waters Alliance HPLC system, using a

Waters 300 mm Xbridge BEH200 SEC column, and a 100 mM Sodium Phosphate, 200 mM L-Arginine mobile phase (pH 6.8). A protein load of 20 ug per injection was targeted, running at a flow rate of 1 mL/min for 20 minutes and detected at wavelength of 280 nm. The fractionation of ERT1-AF594 was performed on an AKTA Explorer instrument (GE Healthcare) using a HiPrep 26/60 Sephacryl™ S-2000 semi-analytical SEC column (GE Healthcare) to fractionate the samples while flowing at 5 mL/min in 100 mM Sodium Phosphate, 200 mM L-Arginine buffer (pH 6.8). The chromatograms above show that the dispersed, size populations cannot be isolated from one another 100%. However, they do show that the different fractions of interest were significantly enriched for different constituents of the polydispersed population.

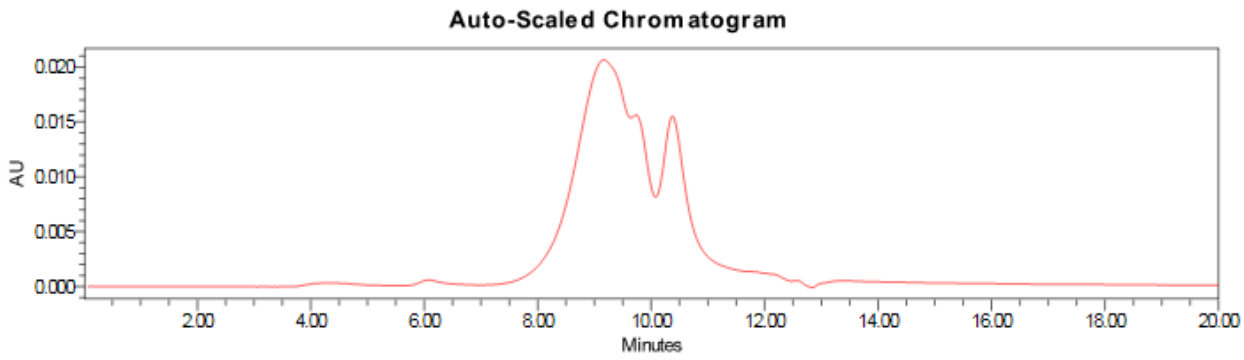


Figure 10: Chromatogram of the unfractionated ERT2-AF488 conjugate run by analytical SEC and detected at 280 nm. Analytical SEC for ERT2-AF488 was performed on a Waters Alliance HPLC system, using a Waters 300 mm Xbridge BEH450 SEC column, and a 100 mM Sodium Phosphate, 200 mM L-Arginine mobile phase (pH 6.8). A protein load of 20 ug per injection was targeted, running at a flow rate of 1 mL/min for 20 minutes and detected at wavelength of 280 nm.

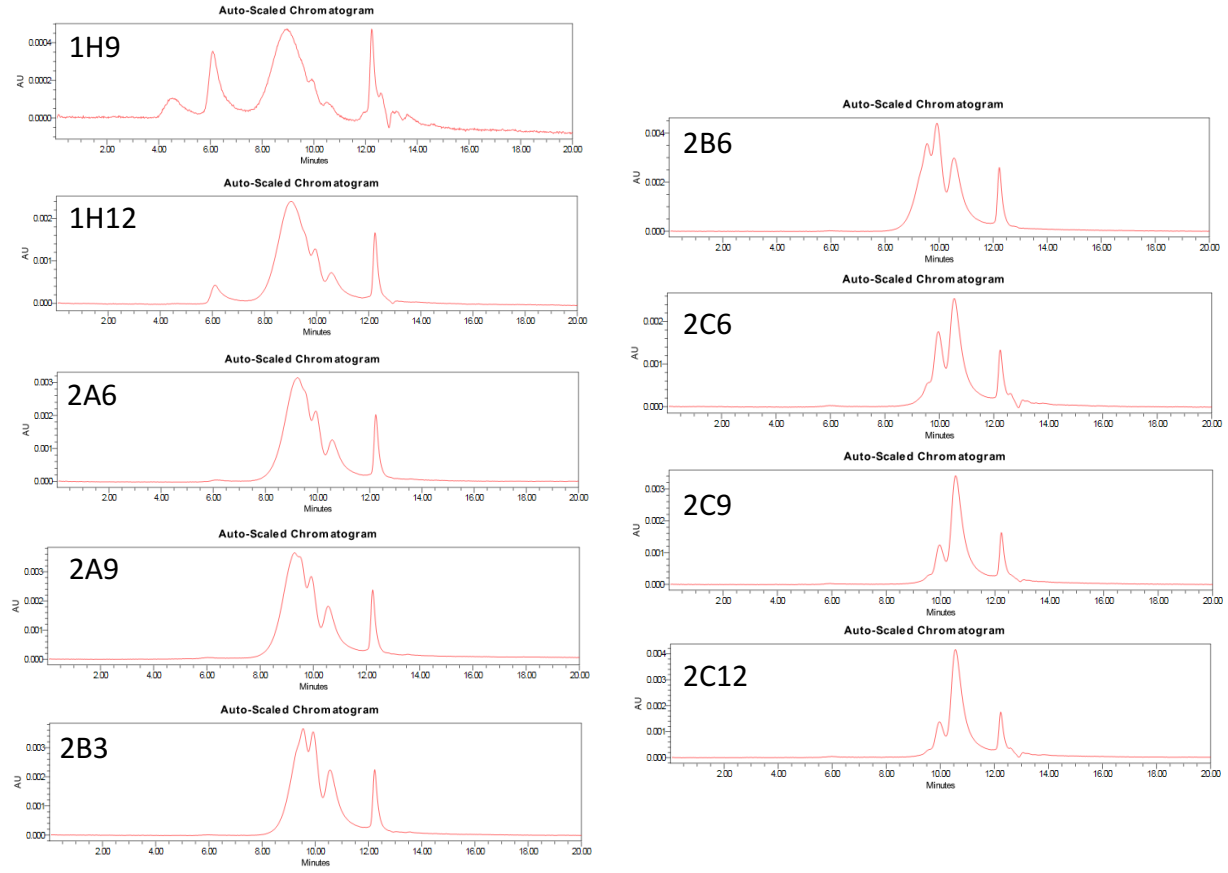


Figure 11: Chromatograms of each fraction of interest for ERT2-AF488 run by analytical SEC. Analytical SEC for ERT2-AF488 was performed on a Waters Alliance HPLC system, using a

Waters 300 mm Xbridge BEH450 SEC column, and a 100 mM Sodium Phosphate, 200 mM L-Arginine mobile phase (pH 6.8). A protein load of 20 ug per injection was targeted, running at a flow rate of 1 mL/min for 20 minutes and detected at wavelength of 280 nm. The fractionation of ERT2-AF488 was performed on an AKTA Explorer instrument (GE Healthcare) using a HiPrep 26/60 Sephacryl™ S-2000 semi-analytical SEC column (GE Healthcare) to fractionate the samples while flowing at 5 mL/min in 100 mM Sodium Phosphate, 200 mM L-Arginine buffer (pH 6.8). The chromatograms above show that the dispersed, size populations cannot be isolated from one another 100%. However, they do show that the different fractions of interest were significantly enriched for different constituents of the polydispersed population.

Chapter IV

Discussion

Preliminary Data:

The preliminary work performed to probe the effects of polydispersity on degree of labeling was primarily performed on a Waters Alliance HPLC system using an SEC method created for that purpose. The earliest work was done in a 100 mM sodium phosphate mobile phase (at a pH of 6.8). However, after some development work, 200 mM L-Arginine was added to the mobile phase to prevent any hydrophobic (non SEC) interactions from interfering with proper separation exclusively by size.

Repeatedly, during this testing, it was noted that the fluorescent conjugates consistently showed a noticeably lower level of fluorescent response coming from higher molecular weight peaks when detected at the proper fluorescent excitation and emission wavelengths (Refer to Figure 3 and Figure 4B). It was during these early experiments that the basic idea for this research and eventual thesis began to develop: That the aggregated population of a protein in a conjugation reaction has the same number of chemical interactions as the less aggregated constituents. This property is presumed to lead to differing degrees of labeling across the polydispersed conjugate population. Because the fluorescent conjugates produced were readily visualized by simple fluorescent detection, it was decided that the study of this phenomena would be most effectively elucidated by using fluorescent conjugates. There are many other common conjugate molecules used in ligand-binding assays, but few are as easily detected and

visualized as fluorescent tags. Thus, for the purposes of this study, fluorescent conjugates were produced to best determine any differences in DOL due to polydispersity.

We visualized the conjugation reaction as occurring in a vast empty space, where each dilute particle, regardless of its number of subunits, has the same average number of chemical interactions with the dye molecules. For example, a portion of the dispersed population composed of four protein subunits binds the same number of conjugate, dye molecules as the population consisting of a single subunit; for simplicity's sake, let us assume each receives a single dye label. If this is the case, when we compare the 280 nm signal to the levels of fluorescent response coming from the different, polydispersed populations of the conjugate, we see a vastly unequal response. If the reaction occurs in the manner assumed above, the aggregate with four subunits would result in a DOL of 0.25 while the monomer, while a single protein subunit, would have a DOL of 1.0.

Conjugation of ERT 1 and 2:

Based on the preliminary data collected, two ERT drugs were chosen as promising candidates to study the theory underpinning this thesis. Both proteins had previously proven to be prone to aggregation during conjugation reactions, with Figures 7 and 9 clearly showing how heavily aggregated and dispersed these populations of conjugate molecules became after labeling. The ERT1-AF594 and ERT2-AF488 conjugates used for the purposes of this thesis were identified based on this shared property. When performing the conjugation of ERT1 to AF-594, the final product was desalted and buffer exchanged using an AKTA instrument (refer to Figure 5), while the product of the ERT2-AF488 was dialyzed over several days. This was

difference was due to the established procedures for producing these conjugates, which called for the two different methods of buffer exchange and free-dye removal.

Fractionation and Collection of Samples:

To confirm that these polydispersed populations did, in fact, have different degrees of labeling, the two lots of fluorescent conjugates were fractionated, by particle size, into 0.5 mL fractions using an AKTA Explorer instrument with a GE HiPrep 26/60 Sephacryl™ S-2000 semi-analytical SEC column. The fractions of interest were chosen based on their positions under the detected peaks (refer to Figures 6 and 7). Although we were not able to fully separate each of the size populations from one another, Figures 8 and 10 help us understand how significantly enriched the fractions were for the different size populations. This being the case, it would seem safe to assume that any phenomena or trends observed would be even clearer if a better separation technique was identified and optimized to suit our specific purposes.

It should also be noted that when fractionating a sample by size, any of the aggregates or low molecular weight species that are only present in relatively small amounts, will become very dilute when fractionated out of the sample. If our analytical SEC shows that a sample is 5% aggregates, we can only hope to collect 5% of the protein in a given fraction. If the fractionation is run with 5 mg of conjugate protein, at best we will collect 0.25 mg of aggregate. This can be a complicating factor when determining degree of labeling as small values in the detected protein concentration can prove to be difficult to work with as small differences in protein concentration can have relatively large effects on the calculated DOL when the concentrations are very low. If this is the case, it becomes much more important to accurately determine the precise value of that low protein concentration.

Determination of Degree of Labeling for Polydispersed Populations:

In our attempt to definitively calculate the differing DOLs of the polydispersed population in our conjugates, multiple replicates and dilutions of each fraction were measured using a NanoDrop 2000 spectrophotometer instrument and averaged to calculate the degrees of labeling for the conjugate population in each fraction tested. The reference to "corrected absorbance" in this portion of the testing alludes to the fact that we are correcting the raw absorbance values obtained from the instrument based on the dilution factor of the sample being tested. Tables 2 and 3 provide very strong evidence that there is, in fact, a significant difference in DOL between the different constituents of a polydispersed, protein-conjugate population. The highest molecular weight population found in ERT1-AF594 had only 17.8% the amount of labeling as the most labeled population from fraction 1F1. Further, as the particle size decreases (meaning less aggregated, or composed of fewer subunits in this case), the DOL shows a clear, rising trend. The DOL data is supportive of the mechanism proposed here, which leads to differing degrees of labeling in polydispersed protein populations, and as presented in this thesis.

Once we understand the principals of the labeling reaction presented in this thesis, the trend of increasing DOLs as the number of protein subunits decreases is very easy to understand. We are simply seeing the effect of each *particle* in the reaction (not each subunit) having the same average number of potential binding interactions during conjugation. The particles themselves end up roughly equally labeled, but because they have differing numbers of subunits (or literally, different amounts of protein) their degrees of labeling can differ vastly. The ratio of protein concentration vs. dye absorbance for the aggregates is slightly different than the ratio we

find for the less aggregated population. For ERT1-AF594, this leads directly to a different, calculated DOL when used in the equation below:

DOL = $[A_{590}]$ ERT1-AF594 x (Mol. Weight of ERT1) / [ERT1-AF594] mg/mL / ε of AF594

Mol. Weight of ERT1 = 70,000; the absorbance coefficient (ε) of AF594 = 94,000

There is an inverse relationship between protein concentration and absorbance. As the protein concentration increases relative to dye absorbance, the resulting DOL we calculate will be lower. Taken with our preliminary data (refer to Figure 3 and 4B), where we saw a vastly different amounts of fluorescent response coming from the different size constituents of the two conjugate samples, the data from this experiment provides strong confirmatory evidence supporting our theory that polydispersity aggregation affect the degree of labeling in protein conjugation reactions. Further, knowing that the level of separation obtained during the fractionation step was imperfect—and the resulting fractions were only enhanced for certain size constituents, as opposed to isolating these constituents completely—likely indicates that the differences in DOL between the size populations is probably more pronounced than the data presented here would indicate.

Confirmation of Fraction Content by Analytical SEC:

This portion of the study was done as a final, confirmatory step in order to prove that the fractions collected during fractionation on the AKTA Explorer contained, or were enriched for, conjugate particles of a different size. As we had hoped, the data obtained showed with relative clarity that the fractions were considerably enriched for different and separate size populations. For example, fraction 1D2 in Figure 8 shows an excellent level of isolation/enrichment of the highest molecular weight constituent of the sample. The high level of isolation obtained in this

fraction may have contributed to how clearly the trend in differing DOLs was shown in the fraction (only 17.8% of the most labeled species in that conjugate sample).

Conclusions:

When interpreted correctly and taken together, the data presented in this thesis provides strong evidence for the theory that polydispersity in a protein population leads to differing degrees of labeling upon conjugation. The proposed mechanism presented here, which leads to differing DOLs—that all protein *particles* in the reaction have the same number of chemical interactions with conjugate molecules, regardless of their number of *subunits*—appears to hold true after testing. Although this theory was tested only with fluorescent conjugates (due to the limitations in our ability to accurately detect and quantify the DOL of protein conjugates made using other labeling/reporting formats), nothing about the data would lead us to believe that this scenario would not hold true for a variety of conjugates. An important question remains however: How this discrepancy in DOL might affect a ligand-binding assay it is used for?

For example, the ERT1-AF594 conjugate is a critical reagent used in a cellular uptake pK assay. As the cell accumulates the enzyme, it also accumulates fluorescence, which can then be read to determine the levels of uptake. However, if the enzyme population used as a reagent is not equally labeled, the accuracy of our results then become questionable, and the critical reagent we are depending on to provide solid results could actually be functioning sub optimally in the assay itself. Assuming the cells being assayed can take up the less labeled, more aggregated enzymes, we must assume those cells will not display the proper amount of fluorescent response. If the cell takes up an aggregate with five subunits, instead of showing five corresponding units

of fluorescence (one for each enzyme absorbed), we would see only a single unit of fluorescence, leading us to believe that cell has taken up only a single enzyme. The more severe the aggregation is during the conjugation reaction, the more severe the discrepancy in that conjugate's DOLs and the more pronounced the error in the signal level detected becomes. These effects could vary in intensity based on the reagent and the assay format being used, but would consistently reduce the accuracy and precision that the assay is capable of achieving. As discussed earlier, the quality of critical reagents will directly affect the quality of the ligand-binding assays they are used for. Thus, any reduction in the quality and uniformity of our critical reagents can be expected to have deleterious effects when used in an assay.

With this in mind, how can we reduce or prevent this phenomenon from occurring during conjugation? What can be done to help make our DOLs more uniform across a dispersed protein population? According to the labeling mechanism presented in this work, anything that can be done to reduce protein aggregation in the reaction should also reduce the discrepancy found in the conjugate population's DOL. One potential approach to reducing the aggregation which takes place during the conjugation is to add a small amount of surfactant to the reaction to reduce the protein's propensity to clump together.

For example, performing the conjugation reaction in the presence of 0.01-0.05% Tween-80 could prove beneficial, as it should reduce the number of aggregates formed, and limit the amount of polydispersity in the final product. Another approach to help reduce aggregation would be to more carefully control and limit the extremity of the pH used during the conjugation, as significant and/or rapid changes in the pH of a protein in solution can lead to the formation of aggregates. A third, slightly different approach might consist of employing some form of post-conjugation processing and "clean-up"—such as the separation and fractionation steps presented

earlier in this paper—to remove the more aggregated and less labeled population. We could thereby enhance the population for a more equally labeled and narrower set of polydispersed conjugate proteins. In addition to the approaches mentioned here, it is very likely that there are other alternative approaches that would prove effective in producing critical reagents with more uniform labeling; such approaches and their effects could be probed in a future set of related experiments.

In regards to future experiments of this nature, there are two important improvements that could be made to the testing protocols used here. Due to cost constraints, as the ERT and dye conjugates used in the testing described in this work are either very expensive, very difficult to procure, or both, we were obliged to produce and work with smaller batches of conjugate than we may have otherwise liked. Creating a much larger batch of conjugate likely would have proved useful, especially when quantifying the degrees of labeling for the conjugate population in each fraction. Because some of the dispersed populations are very dilute in the overall conjugate sample, using more bulk conjugate should assist in determining the precise protein concentration of the most dilute populations more easily and accurately. The method used to circumvent this issue during the testing described here was to test and average multiple sample replicates and dilutions to get the most precise concentration value possible. However, simply having more protein per fraction would have made this step much simpler and more straightforward.

The second, noticeably flawed aspect of the testing performed here was our inability to fully separate the polydispersed populations from one another during fractionation. Without fully isolating the populations, the clarity of the trends identified and presented for each conjugate was very likely reduced from what we might predict to be their true, optimal levels. If

the fractionation had been more complete, possibly by using a different column for the separation or a more optimized set of running parameters, it is assumed that the relationship between aggregation and degree of labeling would be even more pronounced than it was found to be during the testing presented in this thesis.

Although, the level of enhancement attained between the dispersed populations found in the different fractions was more than sufficient to establish the presence of a strong, consistent trend, mitigating the shortcomings identified in the fractionation step to improve separation would likely have produced even more compelling results. I would, however, still consider the testing quite successful, despite the flaws and potential improvements identified. Any future research into this subject, incorporating the changes and improvements noted above, would be very interesting and should, I expect, produce strong supporting data.

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Appendix I

Definition of Terms

Anti-Drug Antibody (ADA): An antibody specifically raised against a biotherapeutic of interest.

Challenge Ratio: The ratio of conjugate to source protein used in a conjugation reaction.

Critical Reagent: "An essential component of a ligand-binding assay whose unique characteristics directly impact assay performance." (Giest et al., 2013).

Degree of Labeling (DOL): The degree of labeling is a value that represents the average number of conjugates, or labels, per unit of protein in a protein conjugate.

Enzyme Linked Immuno Assay (ELISA): A plate-based assay technique designed to detect and quantify substances such as proteins and antibodies.

Fractionation: A separation process where a certain quantity of protein (enzymes, antibodies, peptides, etc.) is divided into smaller quantities, or fractions, based on a gradient.

Immunogenicity: The propensity of a treatment to cause an immune reaction in the patient.

Ligand-Binding Assay (LBA): A ligand-binding assay is an analytical method that uses assay reagents with high binding affinity capable of binding a target analyte in a biological matrix.

Size Exclusion High Chromatography (SEC): An HPLC technique used to separate particles based on size.

Source Protein: A term referring to the unlabeled protein (to be conjugated in a conjugation reaction) prior to its use as a critical reagent in a ligand-binding method.